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Original Article

Inhibition of neddylation plays protective role in lipopolysaccharide-induced kidney damage through CRL-mediated NF-κB pathways

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Abstract: It has been shown that NF-κB signaling path is very effective pharmacological target for the treatment of various inflammatory diseases, including bacterial infection-associated acute kidney injury (AKI), which remains a main cause of disability and death in patients. Notably, IκB, the upstream molecular of NF-κB, plays an important role by inhibiting NF-κB activity, and IκB is regulated by cullin-RING E3 ligases (CRLs)-mediated proteasomal degradation. Therefore inhibition of CRLs-mediated neddylation and degradation of IκB would prevent NF-κB-mediated inflammation. MLN4924, a potent neddylation-inhibiting pharmacological agent, has been shown to have significant protective effects against lipopolysaccharide (LPS)-induced pro-inflammatory cytokine production through restriction of the CRL-mediated NF-κB pathway. However, it is still unclear whether MLN4924 plays a protective role through its anti-inflammatory properties in sepsis-induced AKI. In the current research, we explored whether MLN4924 have anti-inflammatory action in LPS-induced AKI mice. Our results show that MLN4924 dramatically decreased the cytotoxicity of LPS and inhibited LPS-induced synthesis and release of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, in HK2 cells, a renal tubular cell line. In addition, MLN4924 inhibited Nedd8-activating enzymes, which broke the process of cullin proteins neddylation and subsequent CRL target proteins degradation. The MLN4924-induced degradation of CRL attenuated the phosphorylation modification of IκB and IKK-α/β and blocked the nuclear translocation of P50-NF-κB and P65-NF-κB in HK2 cells under LPS stimulation. Finally, our *in vivo* results show that MLN4924 protected against LPS-induced AKI at relatively low doses. Collectively, these results suggest that pharmacologically blocking neddylation by MLN4924 results in the suppression of pro-inflammatory cytokines generation through the CRL/NF-κB pathway in LPS-stimulated HK2 cells, and attenuated renal inflammation in LPS-induced AKI.

Keywords: Neddylation, MLN4924, anti-inflammatory effect, NF-κB pathways, acute kidney injury

Introduction

Renal dysfunction and acute kidney injury (AKI) play a key role in the morbidity and mortality in sepsis patients [1]. The research and development of new agents for the treatment of AKI has great potential for clinical use [2]. However, up-to-date, there is still lack of effective drugs for septic AKI.

It has been shown that nuclear factor-κB (NF-κB), an omnipresent pro-inflammatory transcription factor in mammalian cells, plays a pivotal role in the regulation of pro-inflammatory factors production, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and other related genes response during lipopolysaccharide (LPS) stimulation [3, 4]. The IκB kinase enzyme complex is part of the upstream NF-κB signal transduction cascade, the IκBα protein inactivates the NF-κB transcription factor by masking the nuclear localization signals of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm. It has been proved that the deterioration of the IκBα was dominated by ubiquitylation, and further this ubiquitylation relies on neddylation. Cullins are scaffold proteins that play a support role in the multiunit Cullin-RING E3 ligases (CRLs) [5]. Neddylation is the binding of target proteins with the ubiquitin-like protein neural precursor cell expressed developmentally

down-regulated 8 (NEDD8) [6, 7]. Importantly, cullins are also well-characterized targets of neddylation, therefore CRLs-mediated neddylation and degradation of IκB would promote NF-κB-mediated inflammation.

The process of neddylation is similar to ubiquitination, while it relies on the reactions of the its own enzymes, such as Nedd8-activating enzyme E1 (NAE1), Nedd8-conjugating enzyme E2 (Ubc12), and Nedd8-E3 ligases [8]. Pevonedistat (MLN4924) is a first-in-class inhibitor of NAE1 [6, 9, 10]. After their binding at the active site, the covalent Nedd8-MLN4924 will block the neddylation pathway [10]. Consequently, the Cullin activation is blocked, leading to the accumulation of many CRL substrates, including IκB-α, thereby inhibiting NF-κB activity [11, 12]. Indeed, MLN4924 has been studied to decay LPS-induced pro-inflammatory cytokines generation in macrophages and rebate IFN-β expression in respone to TLR3/4 and retinoic-acid-inducible gene-I stimulation [13, 14]. These results suggest that the neddylation pathway plays a pivotal role in the immune regulation in hypercytokinemia and imply that the neddylation inhibitors may be used as effective anti-inflammatory agents. Based on these findings, this research work was aimed to investigate whether MLN4924 could attenuate LPS-induced AKI through the inhibition of the CRL/NF-κB pathway.

Materials and methods

AKI animal model

All animal experiments were approved by the Ethic Committee on Animal Care of Ruijin Hospital, School of Medicine, Shanghai Jiaotong University. Twenty-five male C57BL/6 mice (10 weeks old) were used including five treatments: vehicle (saline) (n=5), LPS (n=5), LPS+MLN4924 (0.05 mg/kg, n=5), LPS+MLN4924 (0.2 mg/kg, n=5) and LPS+MLN4924 (0.5 mg/kg, n=5). LPS (10 mg/kg; Sigma, USA) and MLN4924 (TargetMol, USA) were injected through the tail vein. For the LPS+MLN4924 group, LPS and MLN4924 were administered simultaneously. Mice treated with saline were used as control. After intervening for 24 hours, the animals were sacrificed by CO_2 asphyxiation followed by the blood samples and renal tissues collection, which were used for biochemical analysis and morphologic studies, respectively.

Cell culture

HK2 cell line was purchased from ATCC (CRL-2190, USA) and cultured in cell incubator under 5% CO_2 at 37°C in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). LPS was dissolved using sterilized phosphate-buffered saline.

Cell viability test

Cell viability was examined by a CCK-8 test. Briefly, HK2 cells were seeded in 96-well cell culture plates, and treated with MLN4924 or/and 2 μg/ml LPS. After treating for 24 hours, the medium was discarded and changed with 100 μl fresh DMEM medium which contains 10 μl of CCK-8 solution (Cell Counting Kit-8, Dojindo, Japan) and incubated for 4 h at 37°C. Then the absorbance was measured at 450 nm with a microplate reader (Infinite F50, TECAN, Switzerland). After subtracting the blank absorbance values, the subsequent analyses were conducted. The growth inhibition of HK2 cells was calculated as the percent viability compared with the control group (100%). The effect of MLN4924 on cell viability at the selected concentration was also analyzed by using a Live/Dead Cell kit (Life Tech, USA). After a 72-h culture, cells were treated with 500 μL of prepared dye for 10 min in the incubator and detected by fluorescence microscopy (Zeiss, Axio M1, Germany).

Cytokine assays

The creatinine concentrations of mice blood were determined using an Auto Analyzer (Roche Diagnostics, Germany). The cell culture medium were collected and the concentration of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 were measured by using the enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc, USA). Briefly, HK2 cells were plated in 24-well plates, with or without treatment of MLN4924 (10, 50 and 100 nM) and 2 μg/ml LPS for 24 h. Then, a 100 μl aliquot of supernatant culture medium was collected to determine the cytokine concentrations by the ELISA kits.

Histology and immunohistochemistry analysis

For histologic analysis, the mice were sacrificed and kidney tissues were fixed with 4% buffered

paraformaldehyde for 24 hours. Then they were embedded by using paraffin, and 5 μm thick continuous sections were obtained. H&E staining were carried out and the histologic changes were scored with the criterion in previous studies. According to the percentage of tubules that displayed damaged change they were scored as follows: no damage scores 0, 10% of tubules were damaged scores 1, 11-25% damage scores 2, 26-45% damage scores 3, 46-75% damage scores 4, and over 76% of damage scores 5 [15, 16]. For immunohistochemistry analysis, a mouse monoclonal against the active form of the p65 subunit of NF-κB (specifically against the NLS) (Chemicon, Harrow, UK) was used to detect its nuclear localization at a dilution of 1:200.

Confocal microscopy

After intervention, the HK2 cells were collected fixed in 4% formaldehyde. Then cells were permeabilized with 0.3% Triton X-100 for 0.5 h and blocked with 2% BSA. Next, HK2 cells were first incubated with anti-P65-NF-κB antibody (1:100, Cell Signaling Technology, USA) at 4°C overnight and then incubated with the FITC-conjugated goat anti-mouse IgG secondary antibody (1:400, Cell Signaling Technology, USA). And DAPI was used to stain cell nuclei. Finally the immunofluorescence images were obtained by using confocal microscopy (Zeiss LSM800, Germany). And images were produced using Zeiss Microscopy (Axio Imager, Germany).

Quantitative real-time PCR

Total RNA of the HK2 cells was obtained using TRIzol (Ambion) according to the manufacturer's specification. Complementary DNA was obtained from 1 μg of total RNA by using the special PrimeScript RT reagent kit (TaKaRa, Japan) according to the manufacturer's direction. Real-time PCR was performed using a SYBR Green qRT-PCR kit (TaKaRa, Japan) and an ABI Step One Plus Real-Time PCR System (Applied Biosystems, USA). β-actin was set as the internal control. Each test was performed in triplicate. The primers used were listed as follows: β-actin sense, CATGTACGTTGCTATCCAGGC and antisense, CTCCTTAATGTCACGCACGAT; TNF-α sense, ATGTCTCAGCCTCTTCTCATTC and antisense, GCTTGTCACTCGAATTTTGAGA; IL-1β, sense, CAACTGTTCCTGAACTCAACTG and antisense, GAAGGAAAAGAAGGTGCTCATG; and IL-6, sense, CTGGTCTTTTGGAGTTTGAG and antisense, GGTCAGGGGTGGTTATTG. The relative mRNA expression changes were calculated by using the 2-ΔCt method.

Cytosolic and nuclear protein preparation

Whole proteins of HK2 cells were obtained basing on the routine procedure in our lab by using cell lysis buffer. The buffer is made of 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM PMSF. And the cytosolic and nuclear extracts were obtained using a kit (Beyotime, China) according to the manufacturer's instruction.

Western blot

After treatment, the proteins of HK2 cells were obtained and their concentration were measured by using a BCA kit. The protein samples were first fractionated by SDS-PAGE. Then they were moved to a polyvinylidene difluoride membrane (Millipore, US). Next, they were blocked with 5% skim milk for 60 min and then they were incubated with primary antibodies overnight at 4°C. The primary antibodies used were at dilutions of 1:5000 for β-actin (Sigma), 1:1000 for Nedd8 (Cell Signaling), 1:1000 for IκB-α (Cell Signaling), 1:1000 for phosphorylated IκB-α (Cell Signaling), and 1:1000 for NF-κB p65 antibody (Abcam). After washing with PBST for three times the membranes were treated with HRP-conjugated secondary antibodies (Cell Signaling Technology) for 2 hours and then were visualized by using of an enhanced chemiluminescence detection system as suggested by the manufacturer. HDAC1 and β-actin (Santa Cruz, CA, USA) were set as internal controls. The expression of the proteins were quantified according to the gray value by using Image-J software.

Luciferase assays

293T cells were transfected with 2 μg of pNF-κB-MetLuc2-Reporter plasmid (Sangon, China) using an Amaxa Nucleofector II apparatus along with the Amaxa Cell Line Nucleofector V Kit (Lonza, Switzerland) in accordance with the

manufacturer's protocol. After transfection, cells were plated onto a 24-well dish (10^5 cells/well) and stimulated with LPS (2 μg/ml) for 24 h, with or without MLN4924. The luciferase activity was measured using the Ready-To-Glow™ NFκB Secreted Luciferase Reporter System according to the manufacturer's protocol. Luciferase activity in the culture media, which was detected and measured using a microplate reader (Infinite F50, TECAN, Switzerland), was normalized to the total protein concentration of the corresponding whole cell lysates.

Statistical analysis

The data were expressed as the means ± SEM, and each test was repeated at least for three times. Measurement of the statistical significance of the difference was taken by way of Student's t-test for two group comparison or one-way ANOVA followed by Tukey's multiple comparison test. The SPSS software was used to process statistical analysis. $P < 0.05$ was set as the statistical significance unless otherwise indicated.

Results

MLN4924 damps LPS-induced cytotoxicity in HK2 cells

We first determined whether MLN4924 could reduce HK2 cell viability since it has been reported that MLN4924 decays the cell viability of acute myeloid leukemia cell lines at 100 nM or higher [17]. As shown in **Figure 1A**, there was no significant difference in cell viability in HK2 cells with or without 10, 20, 50, and 100 nM MLN4924 for 0, 6, 12, 24 or 48 h respectively. However, significantly decreased cell viability was obtained when cells were treated with 200 nM MLN4924 for 12, 24 and 48 h. The Live/Dead assay also revealed that MLN4924 had no cell toxicity at the concentration of 100 nM (**Figure 1C**). In contrast, **Figure 1B** shows that treating HK2 cells with 2 μg/ml of LPS treatment for 24 h significantly decreased their viability, and the effect of LPS was significantly reversed by MLN4924 (20, 50, and 100 nM) in a concentration dependent manner. Therefore, we chose to treat HK2 cells with 100 nM MLN4924 as the highest intervention concentration in the subsequent experiments.

MLN4924 inhibits LPS-induced production of proinflammatory cytokine in HK2 cells

Previous studies have shown that treating HK2 cells with LPS provokes the production of pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6 [11, 18, 19]. We then evaluated whether MLN4924 would exert anti-inflammatory effect. And to make it clear that whether MLN4924 inhibits LPS-induced cytokine expression and external secretion, we collected the culture media of LPS-induced HK2 cells and analyzed the concentration of IL-1β, TNF-α and IL-6 protein. It was found that MLN4924 dramatically reversed the increases of IL-1β, TNF-α and IL-6 induced by LPS when using at a concentration of 50 and 100 nM (**Figure 2A-C**), and no effect on the excretion of these factors when used at a concentration of 10 nM. Similarly, the result of western blot analysis also revealed that LPSinduced excretion of IL-1β, TNF-α and IL-6, and that the effect of LPS was significantly inhibited by MLN4924 (**Figure 2G-K**).

Because LPS could regulate both IL-6 and TNF-α expression at transcription level [20, 21], we then detected that whether MLN4924 could inhibit the transcriptional activation of these pro-inflammatory cytokines induced by LPS. As shown in **Figure 2D-F**, MLN4924 at 50 and 100 nM significantly prevented LPS-induced mRNA increase of these cytokines. These data suggest that MLN4924 at sublethal doses, simultaneously led to a significant decrease of multiple proinflammatory cytokines in LPS-induced HK2 cells at the protein and transcriptional levels.

MLN4924 affects neddylation and subsequent IκB degradation in HK2 cells

Then, we determined whether MLN4924 would inhibit LPS-induced neddylation. As shown in **Figure 3**, Nedd8 antibody detects a Nedd8-conjugated protein band at approximately 90 kDa, the right molecular weight for a neddylated Cullin protein (**Figure 3A**, top panel) [17, 22]. MLN4924 reduced the increased expression of Nedd8-conjugated protein induced by LPS. Furthermore, we also examined the expression of neddylated Cullin1 protein (**Figure 3A**, middle panel), and MLN4924 also prevented LPS-induced reduction [23].

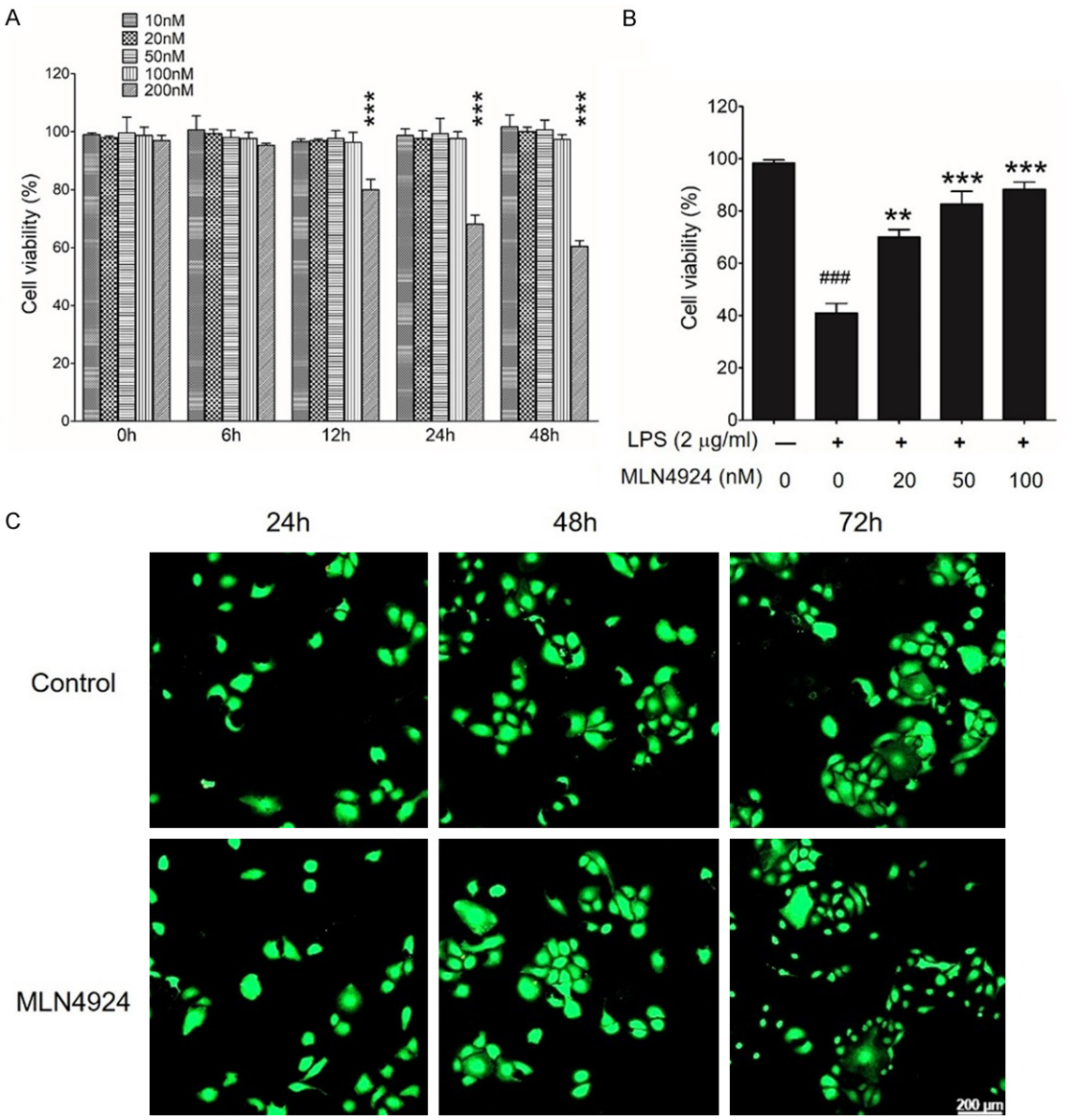


Figure 1. Cytotoxicity test of MLN4924 for HK2 cells. (A) Summarized data showing the cell viability in CCK8 assay in HK2 cells treated with 10, 20, 50, 100, and 200 nM MLN4924 for 6, 12, 24, and 48 h. (B) Summarized data showing the inhibitory effect of 0, 20 50, and 100 nM MLN4924 on LPS-induced cell death. (C) Representative fluorescence images showing the effect of MLN4924 (100 nM) on cell viability in Live/Dead staining. Live cell were green and dead cells were red. There were no dead cells under the MLN4924 (100 nM) treatment. Data were shown are means ± S.E.M. (N=4). ***P < 0.001 vs 10 nM MLN4924 group with the same treatment time in (A). ##P < 0.01 vs the group without LPS or MLN4924; **P < 0.01, ***P < 0.001 vs LPS-treatment group without MLN4924 in (B).

It has been proved that treating HK2 cells with LPS leads to the increase of IκB at both mRNA and protein level [22]. Our results show that when treating with LPS, the IκB protein levels were significantly reduced within 15 min and recovered after 6 h (**Figure 3C**, middle panel). We then detected the change of IκB phosphorylation and found that it is coincide with the decrease of IκB, showing that the levels of phosphorylated IκB increased 1 hour later and returned to low levels 6 h later when treated with LPS (**Figure 3C**, top panel). We then determined whether MLN4924 would promote the degradation of IκB in HK2 cells treated with LPS, since IκB is a target of CRLs. However, when treating HK2 cells with MLN4924 at 100 nM at the same time, the LPS-induced decrease in IκB levels was restrained (**Figure 3D**, middle

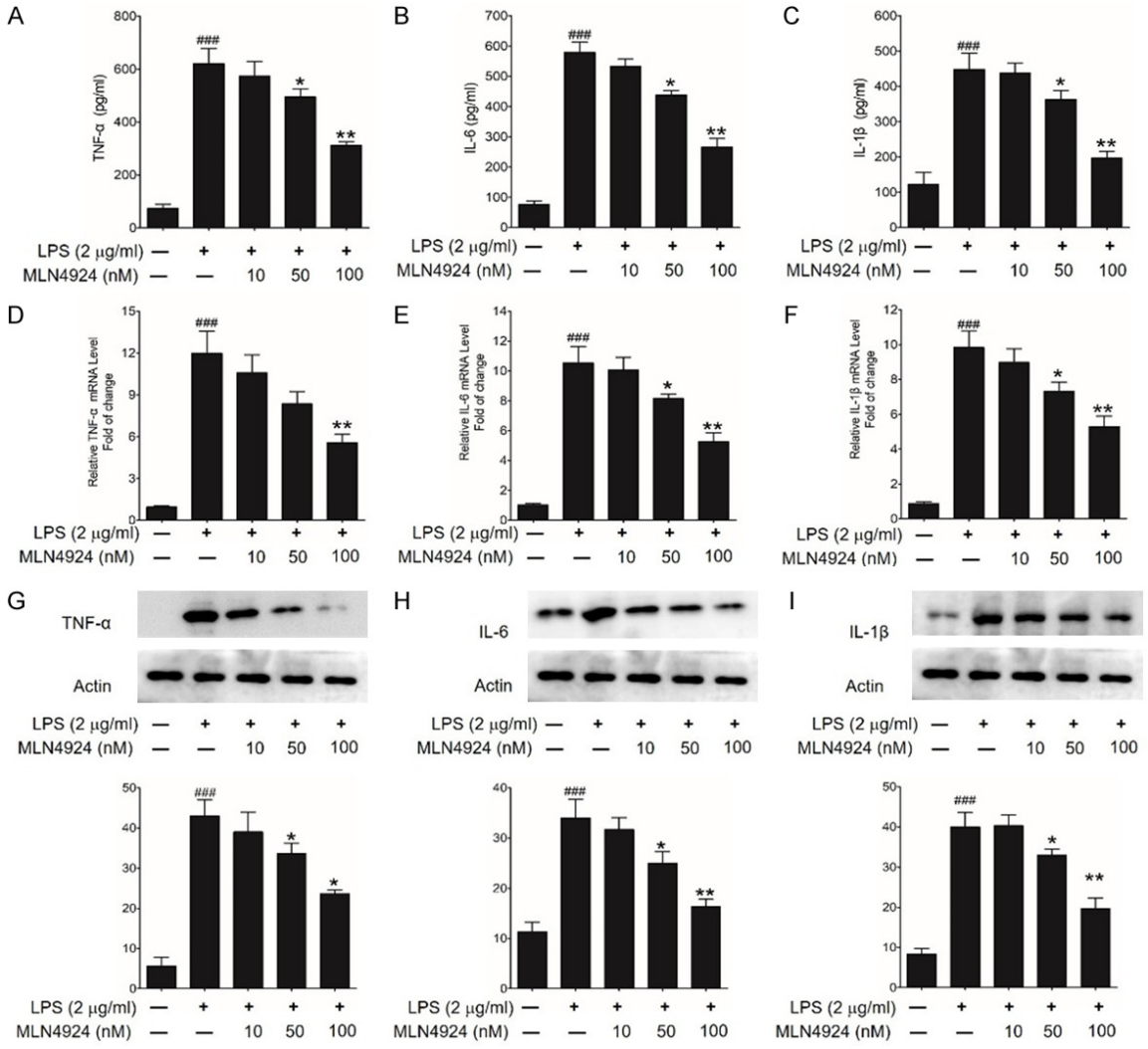


Figure 2. MLN4924 inhibited LPS-induced pro-inflammatory cytokines in HK2 cells. Summarized data showing the inhibitory effect of 0, 10, 50, 100 nM MLN4924 on LPS-induced extracellular cytokines (A-C) and mRNA (D-F) increase in TNF-α, IL-1β and IL-6 in HK2 cells. Representative western blot images (upper panel) and summarized data (lower panel) showing the inhibitory effect of 0, 10, 50, 100 nM MLN4924 on LPS-induced protein increase of TNF-α (G), IL-1β (H) and IL-6 (I). Data were shown are means ± S.E.M. (N=6). ###, $P < 0.001$ vs the group without LPS or MLN4924; *$P < 0.05$, **$P < 0.01$ vs LPS-treatment group without MLN4924; *, P < 0.05, **, P < 0.001 vs LPS-induced group.

panel). In contrast, the levels of phosphorylated IκB increased within15 min and went on elevating for the next 6 h when treating with LPS (**Figure 3D**, top panel). These data indicate that MLN4924 prevented the degradation of phosphorylated IκB in HK2 cells through inhibiting Cullin neddylation and Cullin-mediated of IκB degradation.

MLN4924 affects translocation of nuclear NF-κB in LPS-induced HK2 cells

To further reveal the mechanism of MLN4924 for the protective effect on LPS induced HK2 cells, we next assessed whether MLN4924 could prevent LPS-induced NF-κB transcription. After treating HK2 cells with LPS with or without MLN4924, p65 NF-κB nuclear translocation was determined by western-blot analysis and immunocytochemistry. The result revealed that p65 NF-κB was mainly in cytosolic fraction in non-treated cells, and its distribution was shifted to nuclear fraction after 6 h LPS treatment (**Figure 4A**, top panel). However, treatment with MLN4924 showed prevented LPS-induced shift of p65 NF-κB from cytosolic fraction to nuclear fraction (**Figure 4A**). The shift of p65 NF-κB was further evaluated by immunocy-

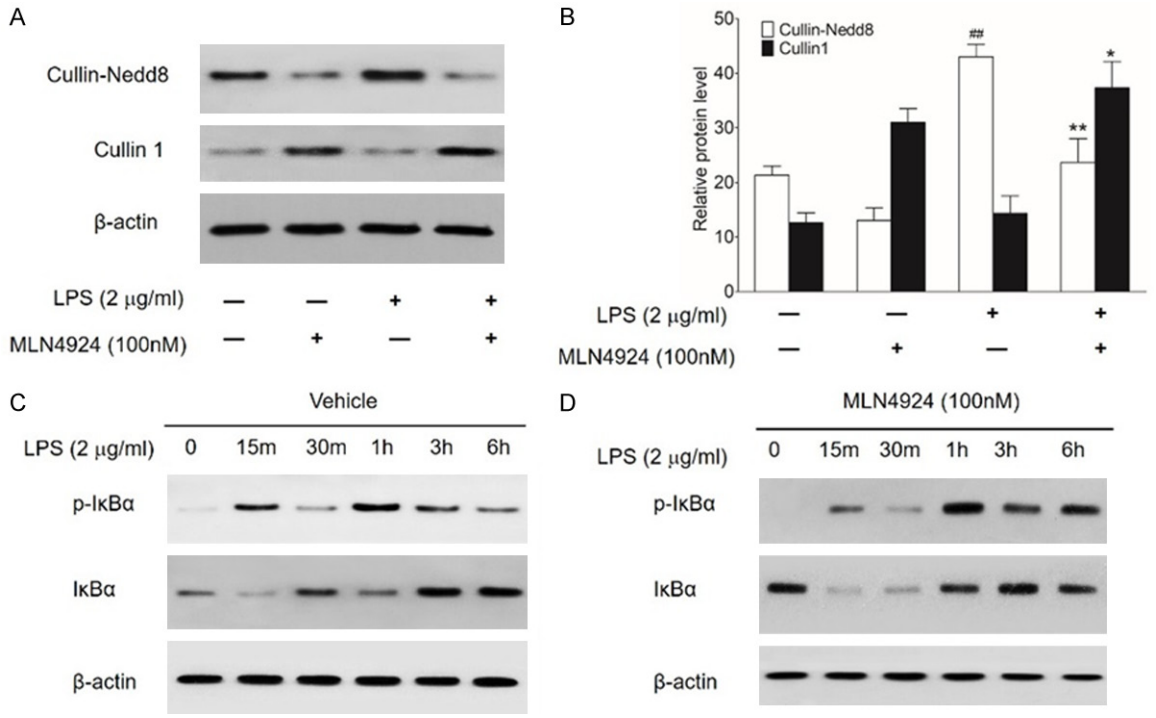


Figure 3. Effect of MLN4924 on neddylation and IκB degradation in LPS-induced HK2 cells after treating with LPS and MLN4924 for 6 h. Representative western blot images (A) and summarized data (B) showing the inhibitory effect of MLN4924 on LPS-induced changes in cullin-Ned88 and Cullin1 in HK2 cells. (C) Representative western blot images showing the time-dependent effect of LPS on p-IkBα and IkBα level in HK2 cells. (D) Representative western blot images showing the inhibitory effect of MLN4924 on LPS-induced changes in p-IkBα and IkBα level in HK2 cells. Values are expressed as the means ± S.E.M. (N=6). ##$P < 0.01$ vs the group without LPS or MLN4924 treatments for Cullin-Nedd8 and Cullin1 respectively; *$P < 0.05$,**P < 0.01 vs LPS-treated group without MLN4924 for Cullin1 for Cullin-Nedd8 and Cullin1 respectively.

tochemistry. As shown in **Figure 5A**, the p65 NF-κB was exclusively stained in cytoplasmic and there was no obvious nuclear accumulation of the protein. However, the accumulation of p65 NF-κB in the nuclear was increased in immunocytochemistry after LPS treatment for 1 h (**Figure 5A**). In addition, there is no significantly difference of p65 NF-κB staining in HK2 cells treated with MLN4924 and non-treated control cells. These results suggest that MLN4924 damps the nuclear translocation of p65 NF-κB induced by LPS in HK2 cells.

Additionally, the effect of MLN4924 on LPS-induced NF-κB transcriptional activity was assessed in 293T cells using a luciferase reporter under control of a promoter containing NF-κB transcription factor binding sites (**Figure 5B**). Luciferase assays revealed that treating transfected cells with LPS caused a 6.2-fold increase in NF-κB transcriptional activity. Furthermore, this LPS-induced increase in NF-κB transcriptional activity was significantly inhibited by treatment of cells with either 100 nM MLN4924, suggesting that MLN4924 inhibits its LPS-induced NF-κB transcriptional activity.

MLN4924 inhibits renal damage and protect renal function through inhibiting NF-κB activation in LPS-treated mice

When treating the mice with LPS alone, massive kidney damage was observed in the tubules. From the HE staining, cortex tubules manifested cellular necrosis and detachment, brush border destruction, cast formation, tubular vacuolization, and tubule dilation (**Figure 6A**). Then percentage of the damage was also scored (**Figure 6B**). The results revealed that the LPS-induced tubular injury was significantly inhibited by MLN4924 at the middle and high concentration (0.2 mg/kg and 0.5 mg/kg). However, treating with MLN4924 at low concentration (0.05 mg/kg) did not affect the LPS-induced tubular injury. Then we further analyzed the function of kidney by measuring

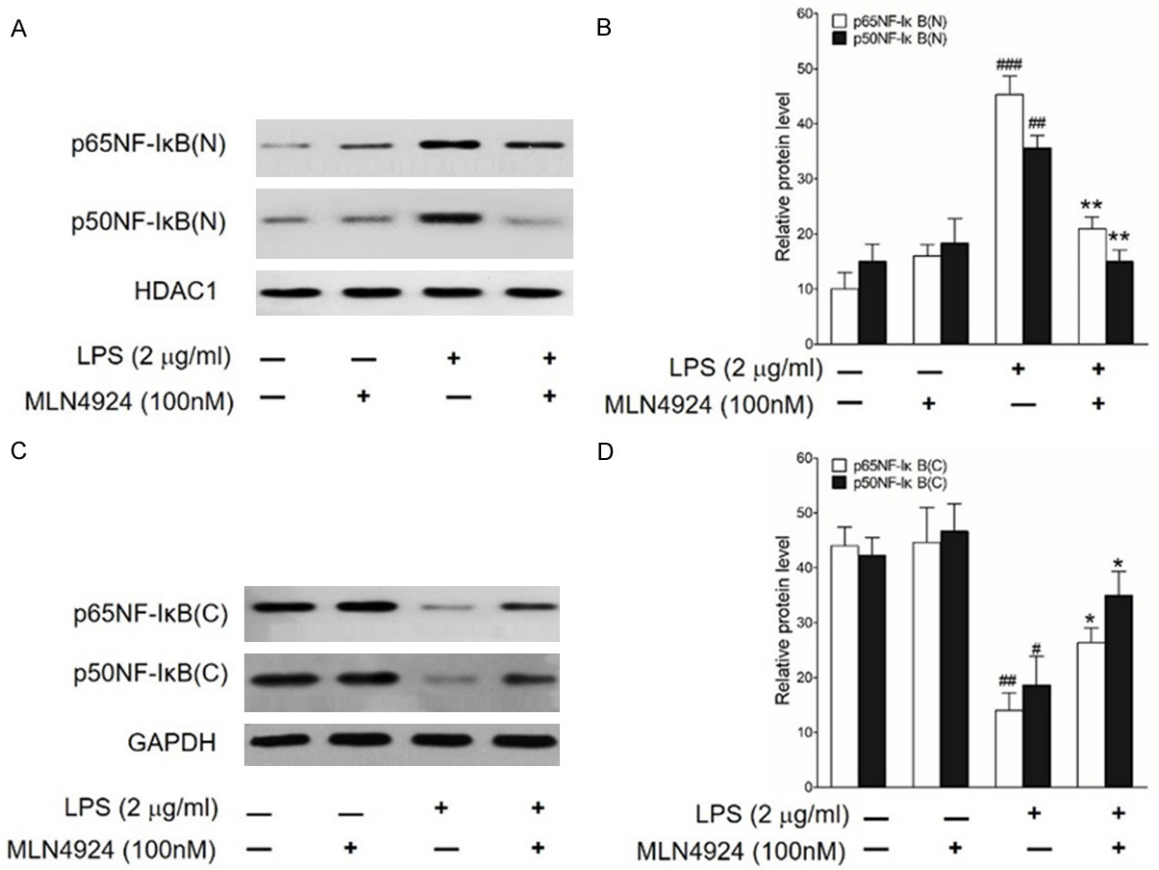


Figure 4. Effect of MLN4924 on NF-κB translocation in LPS-induced HK2 cells after treating with LPS and MLN4924 for 6 h. Representative western blot images (A) and summarized data (B) showing the inhibitory effect of MLN4924 on LPS-induced changes of nuclear P65-NF-κB and P50-NF-κB in HK2 cells. Representative western blot images (C) and summarized data (D) showing the inhibitory effect of MLN4924 on LPS-induced changes of cytosolic P65-NF-κB and P50-NF-κB in HK2 cells. The expression levels of HDAC1 and β-actin were set as internal reference. Data were shown are means ± S.E.M. (N=6). #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 the group without LPS or MLN4924 treatment for p65 NFκB and p60 NFκB respectively; **P* < 0.05, ***P*< 0.01 vs LPS-treated group without MLN4924 for p65 NFκB and p60 NFκB respectively.

serum creatinine. As shown in **Figure 6C**, LPS dramatically increased the serum creatinine levels compared to control, which was significantly prevented by MLN4924. Finally, immunohistochemistry analysis was performed to detect the inhibitory effect of MLN4924 on LPS-induced NF-κB activation in vivo. As shown in **Figure 6D**, there were significant increases in nuclear NF-κB activity in kidney from LPS-treated mice. And this increase was significantly inhibited by high concentration of MLN4924 (0.05 mg/kg). These results suggest that MLN4924 inhibited renal damage and protect renal function through inhibiting NF-κB activation in LPS-treated mice.

Discussion

Septic AKI accounts for dramatic disability and mortality clinically. However, currently there are still no effective treatment therapies for this disease. In this research, we proved that MLN4924, a cancer therapy agent, plays a protective role in LPS-induced AKI. It's well known that the inflammatory response is responsible for the initiation and development of renal injury, including sepsis-induced AKI and other chronic kidney disease such as lupus nephritis (LN) [24, 25]. The generation of numerous inflammatory molecules, including cytokines (TNF-α, IL-1β and IL-6), had been proved to accelerate the development of these diseases. In this study, we revealed that MLN4924 decreased the synthesis and production of these inflammatory cytokines in response to LPS stimulation, both at the protein and transcriptional levels.

The inflammatory response during the pathogenic process in sepsis-induced AKI is mainly

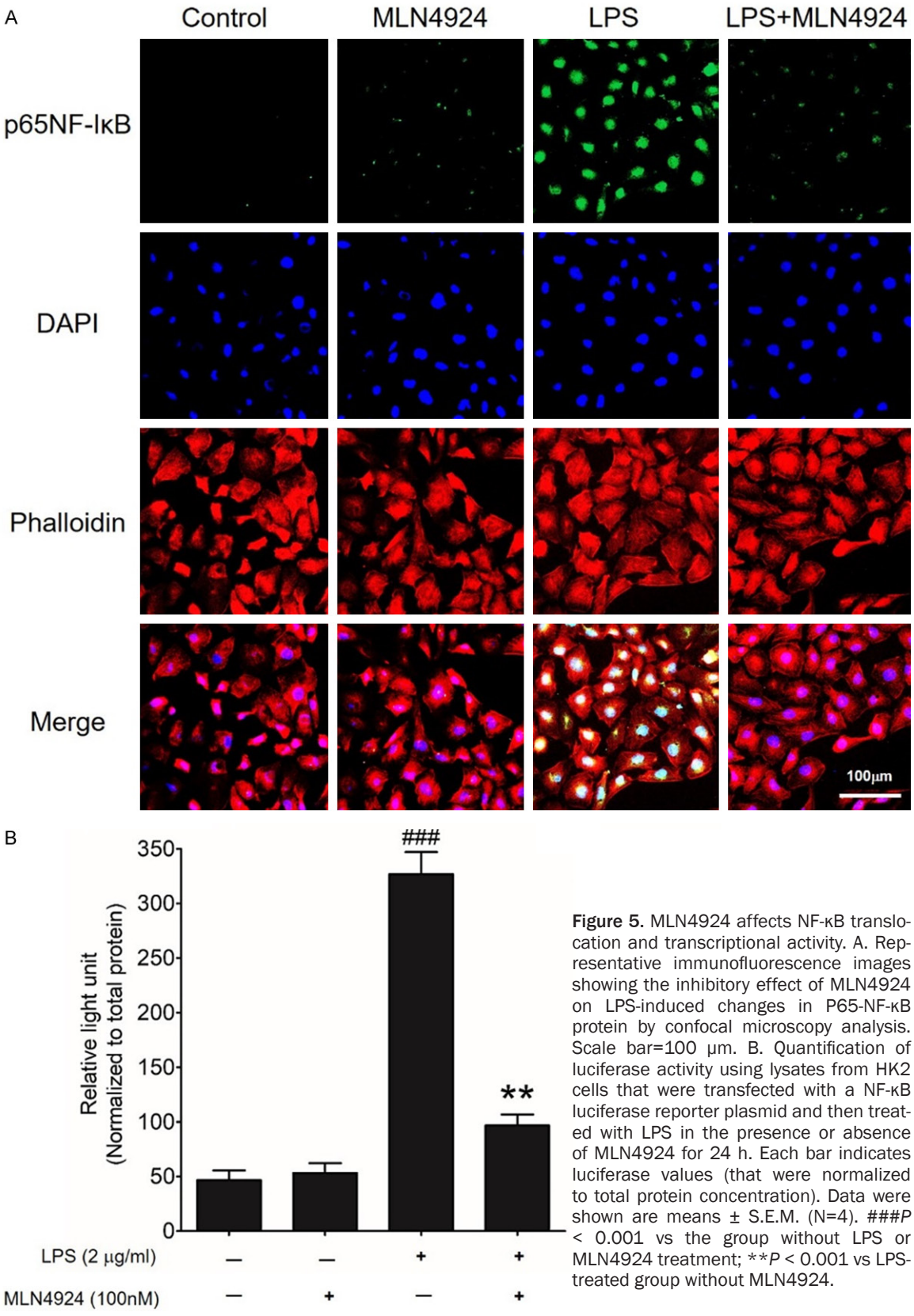
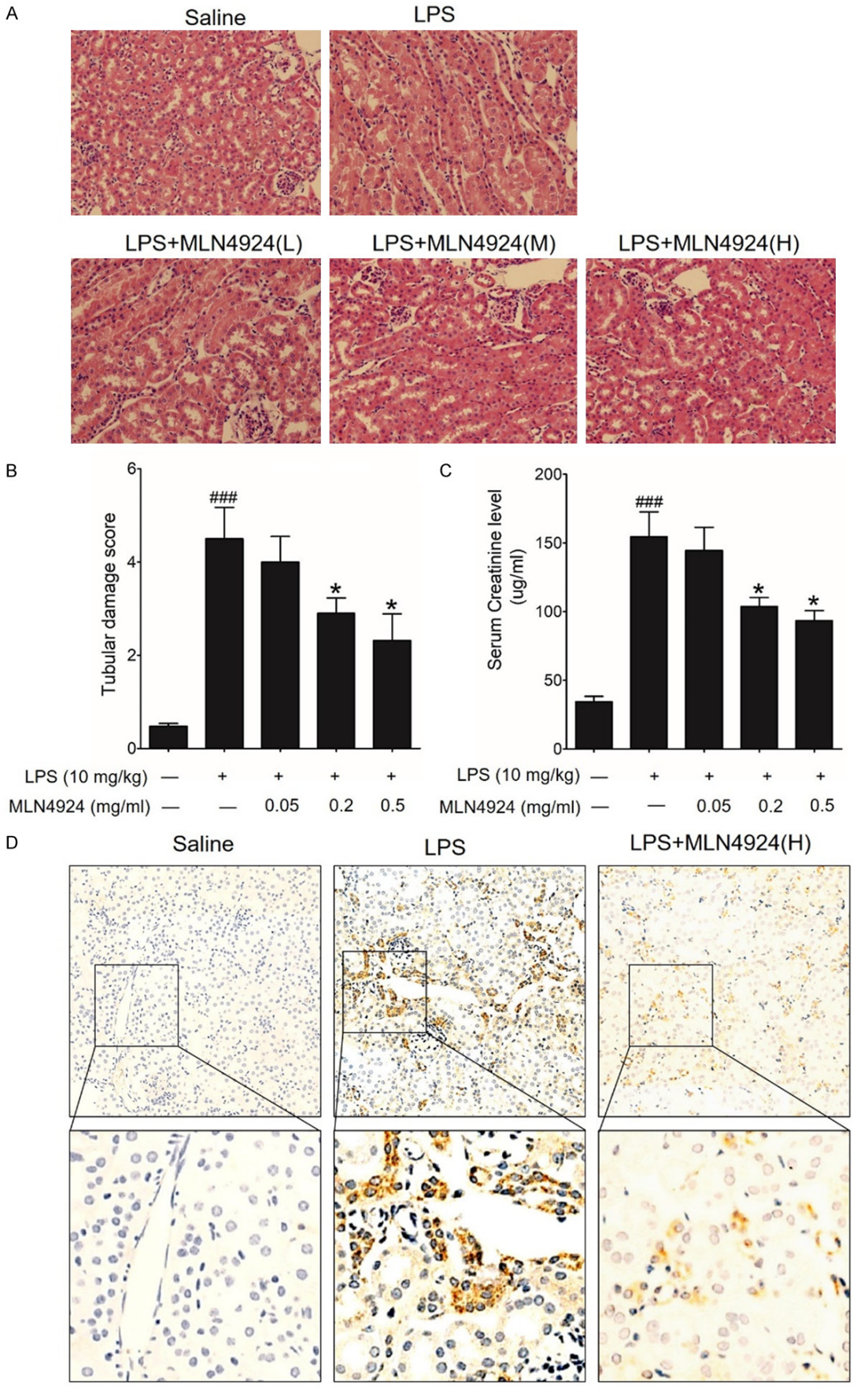


Figure 5. MLN4924 affects NF-κB translocation and transcriptional activity. A. Representative immunofluorescence images showing the inhibitory effect of MLN4924 on LPS-induced changes in P65-NF-κB protein by confocal microscopy analysis. Scale bar=100 μm. B. Quantification of luciferase activity using lysates from HK2 cells that were transfected with a NF-κB luciferase reporter plasmid and then treated with LPS in the presence or absence of MLN4924 for 24 h. Each bar indicates luciferase values (that were normalized to total protein concentration). Data were shown are means ± S.E.M. (N=4). ###P < 0.001 vs the group without LPS or MLN4924 treatment; **P < 0.001 vs LPS-treated group without MLN4924.

depended on the activation of the NF-κB pathway [1, 26, 27]. Previous studies have proved that NF-κB could upregulate the synthesis and secretion of various pro-inflammatory factors,


A
Saline
LPS
LPS+MLN4924(L)
LPS+MLN4924(M)
LPS+MLN4924(H)
B
Tubular damage score
###
*
*
LPS (10 mg/kg) — + + + +
MLN4924 (mg/ml) — — 0.05 0.2 0.5
C
Serum Creatinine level (ug/ml)
###
*
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LPS (10 mg/kg) — + + + +
MLN4924 (mg/ml) — — 0.05 0.2 0.5
D
Saline
LPS
LPS+MLN4924(H)

Figure 6. MLN4924 protected LPS-induced kidney damage in mice. Mice were treated with 10 mg/kg LPS with or without MLN4924 at the low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages for 24 h. Mice treating with saline was set as control. A. Representative hematoxylin & eosin staining images showing the protective effect of low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages of MLN4924 on LPS-induced tubular injury. B. Summarized tubular damage scores showing the protective effect of low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages of MLN4924 on LPS-induced tubular injury. C. Summarized data showing the protective effect of low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages of MLN4924 on kidney function in the serum creatinine measurement. D. Representative immunohistochemistry images showing the inhibitory effect of MLN4924 on LPS-induced changes in P65-NF-κB protein in vivo. Data were shown are means ± S.E.M. (N=5). ###*P* < 0.001 vs the group without LPS or MLN4924 treatment; **P*< 0.05 vs LPS-treated group without MLN4924.

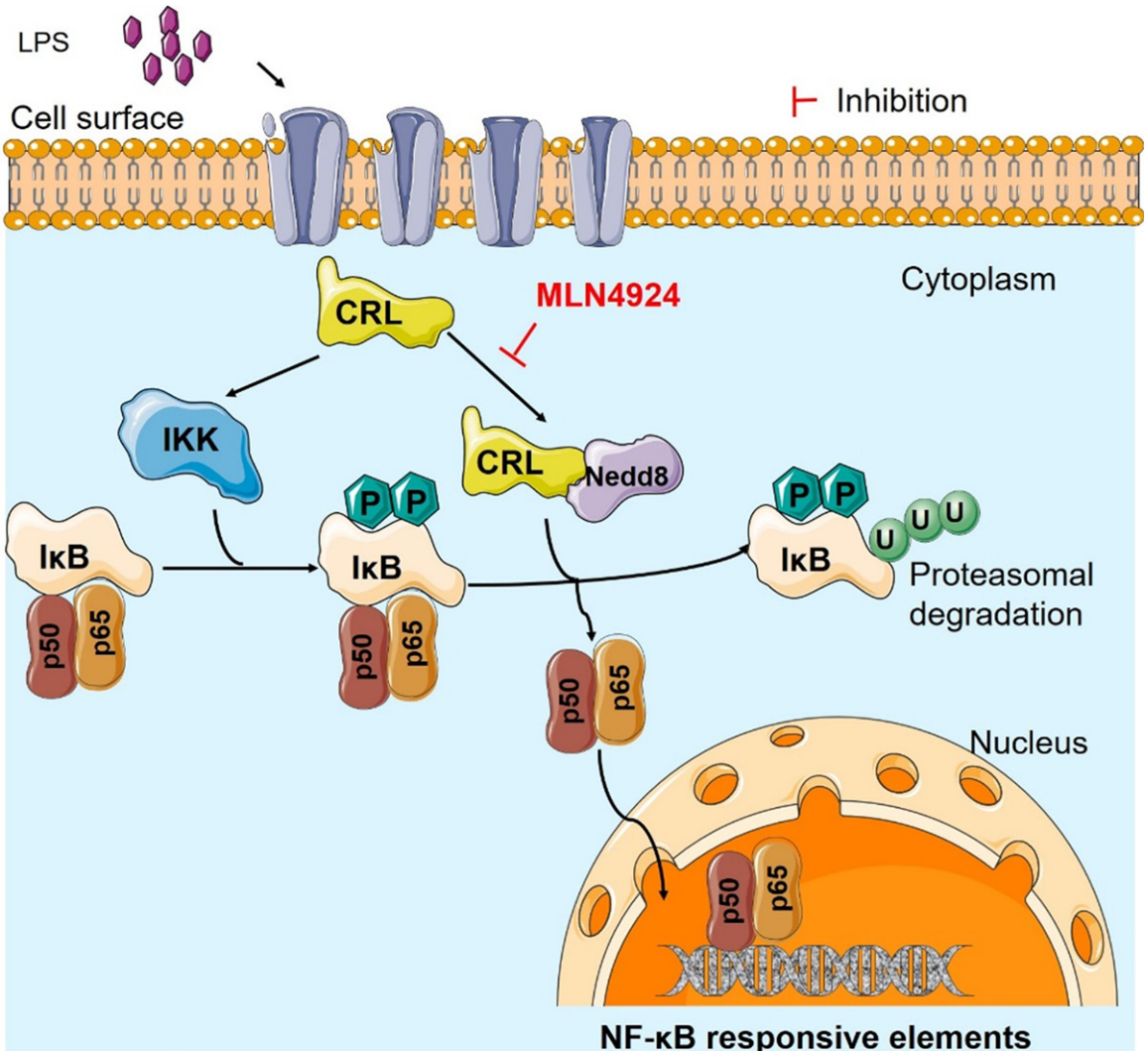

Figure 7. MLN4924 treatment of LPS-induced AKI in a mouse model via inhibition of CRL-mediated activation of the NF-κB pathway.

such as TNF-α, IL-1β, and IL-6. When binding to its inhibitory protein, IκB, the P65/P50-NF-κB protein usually exists in cell cytoplasm in its inactive state. And when stimulating with LPS, the subunits of IKK-α, IKK-β, and IKK-γ will interact with each other to form the IKK complex protein and then phosphorylate the IκB. After phosphorylation, IκB became ubiquitylated and then degraded quickly, which permitted the P65/P50-NF-κB protein to translocate into the nucleus, thereby activating the inflammatory responses [28]. In this study, we have shown that MLN4924 significantly reversed the decreases of IκB protein level and the translo-

cation and transcriptional activation of NF-κB in LPS induced HK2 cells. What is noteworthy is that MLN4924 inhibits LPS-induced cytokine generation through the regulation of neddylation and degradation of IκB. Furthermore, our in vivo study shows that MLN4924 significantly decreased LPS-induced renal damage and improved kidney function in LPS-induced mice. Therefore, MLN4924 might be used as anti-inflammatory agents through inhibiting IKK/IκB/NF-κB pathway.

Notably, the inhibitory effect of MLN4924 on LPS-induced cytokine upregulation was different with dexamethasone in the molecular mechanisms [19]. If this is the case, then MLN4924 might serve as an alternative treatment for diseases concomitant with a defective immune response, including septic shock, chronic liver disease, liver cancer, or metabolic disorders. Furthermore, when using simultaneously, dexamethasone and MLN4924 might exist synergistic effect with better result. Finally, targeting neddylation may server as an effective alternative for those who are resistant to dexamethasone therapy [29-32].

Conclusion

In summary, the present research demonstrated that MLN4924 prevented synthesis and production of inflammatory factors induced by LPS through CRL/NF-κB signaling pathway via inhibition of neddylation (**Figure 7**). The neddylation process might be a novel target for attenuating proinflammatory cytokine production in septic AKI.

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Disclosure of conflict of interest

None.

Abbreviations

AKI, acute kidney injury; NEDD8, neural precursor cell expressed developmentally down-regulated 8; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α.

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References

[1] Zhang D, Li Y, Liu Y, Xiang X and Dong Z. Paclitaxel ameliorates lipopolysaccharide-induced kidney injury by binding myeloid differentiation protein-2 to block Toll-like receptor 4-mediated nuclear factor-κB activation and cytokine production. J Pharmacol Exp Ther 2013; 345: 69-75.

[2] Mayeux PR and Macmillancrow LA. Pharmacological targets in the renal peritubular microenvironment: implications for therapy for sepsis-induced acute kidney injury. Pharmacol Ther 2012; 134: 139-155.

[3] Sadeghi A, Rostamirad A, Seyyedebrahimi S and Meshkani R. Curcumin ameliorates palmitate-induced inflammation in skeletal muscle cells by regulating JNK/NF-κB pathway and ROS production. Inflammopharmacology 2018; 26: 1265-1272.

[4] Rashidian A, Muhammadnejad A, Dehpour AR, Mehr SE, Akhavan MM, Shirkoohi R, Chamanara M, Mousavi SE and Rezayat SM. Atorvastatin attenuates TNBS-induced rat colitis: the involvement of the TLR4/NF-κB signaling pathway. Inflammopharmacology 2016; 24: 109-118.

[5] Luo Z, Pan Y, Jeong LS, Liu J and Jia L. Inactivation of the Cullin (CUL)-RING E3 ligase by the NEDD8-activating enzyme inhibitor MLN4924 triggers protective autophagy in cancer cells. Autophagy 2012; 8: 1677-1679.

[6] Ying J, Zhang M, Qiu X and Lu Y. Targeting the neddylation pathway in cells as a potential therapeutic approach for diseases. Cancer Chemother Pharmacol 2018; 81: 797-808.

[7] Delgado TC, Barbier-Torres L, Zubiete-Franco I, Lopitz-Otsoa F, Varela-Rey M, Fernandez-Ramos D and Martinez-Chantar ML. Neddylation, a novel paradigm in liver cancer. Transl Gastroenterol Hepatol 2018; 3: 37.

[8] Merlet J, Burger J, Gomes JE and Pintard L. Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization. Cell Mol Life Sci 2009; 66: 1924-38.

[9] Swords RT, Erba HP, Deangelo DJ, Bixby DL, Altman JK, Maris M, Hua Z, Blakemore SJ, Faessel H and Sedarati F. Pevonedistat (MLN4924), a First-in-Class NEDD8-activating enzyme inhibitor, in patients with acute myeloid leukaemia and myelodysplastic syndromes: a phase 1 study. Br J Haematol 2015; 169: 534-543.

[10] Nawrocki ST, Griffin P, Kelly KR and Carew JS. MLN4924: a novel first-in-class inhibitor of NEDD8-activating enzyme for cancer therapy. Expert Opin Investig Drugs 2012; 21: 1563-1573.
[11] Muniandy K, Gothai S, Badran KMH, Suresh Kumar S, Esa NM and Arulselvan P. Suppression of proinflammatory cytokines and mediators in LPS-induced RAW 264.7 macrophages by stem extract of alternanthera sessilis via the inhibition of the NF-kappaB pathway. J Immunol Res 2018; 2018: 3430684.
[12] Schwechheimer C. NEDD8-its role in the regulation of Cullin-RING ligases. Curr Opin Plant Biol 2018; 45: 112-119.
[13] Song H, Huai W, Yu Z, Wang W, Zhao J, Zhang L and Zhao W. MLN4924, a first-in-class NEDD8-activating enzyme inhibitor, attenuates IFN-β production. J Immunol 2016; 196: 3117-3123.
[14] Jin J, Jing Z, Ye Z, Guo L, Hua L, Wang Q, Wang J, Cheng Q, Zhang J, Xu Y and Wei L. MLN4924 suppresses lipopolysaccharide-induced proinflammatory cytokine production in neutrophils in a dose-dependent manner. Oncol Lett 2018; 15: 8039-8045.
[15] Miyaji T, Hu X, Yuen PS, Muramatsu Y, Iyer S, Hewitt SM and Star RA. Ethyl pyruvate decreases sepsis-induced acute renal failure and multiple organ damage in aged mice. Kidney Int 2003; 64: 1620-1631.
[16] Brooks C, Wei Q, Cho SG and Dong Z. Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. J Clin Invest 2009; 119: 1275-1285.
[17] Swords RT, Kelly KR, Smith PG, Garnsey JJ, Mahalingam D, Medina E, Oberheu K, Padmanabhan S, O'Dwyer M and Nawrocki ST. Inhibition of NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia. Blood 2010; 115: 3796-3800.
[18] Li L, Liu B, Dong T, Lee HW, Yu J, Zheng Y, Gao H, Zhang Y, Chu Y and Liu G. Neddylation pathway regulates the proliferation and survival of macrophages. Biochem Biophys Res Commun 2013; 432: 494-498.
[19] Chang FM, Reyna SM, Granados JC, Wei SJ, Innis-Whitehouse W, Maffi SK, Rodriguez E, Slaga TJ and Short JD. Inhibition of neddylation represses lipopolysaccharide-induced proinflammatory cytokine production in macrophage cells. J Biol Chem 2012; 287: 35756-35767.
[20] Jang CH, Choi JH, Byun MS and Jue DM. Chloroquine inhibits production of TNF-α, IL-1β and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. Rheumatology (Oxford) 2006; 45: 703-710.
[21] Chanput W, Mes J, Vreeburg RA, Savelkoul HF and Wichers HJ. Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. Food Funct 2010; 1: 254-61.
[22] Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP and Critchley S. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 2009; 458: 732-736.
[23] Wei D, Li H, Yu J, Sebolt JT, Zhao L, Lawrence TS, Smith PG, Morgan MA and Sun Y. Radiosensitization of human pancreatic cancer cells by MLN4924, an investigational NEDD8-activating enzyme inhibitor. Cancer Res 2012; 72: 282-293.
[24] Ding Y, Yang H, Xiang W, He X, Liao W and Yi Z. CD200R1 agonist attenuates LPS-induced inflammatory response in human renal proximal tubular epithelial cells by regulating TLR4-MyD88-TAK1-mediated NF-κB and MAPK pathway. Biochem Biophys Res Commun 2015; 460: 287-294.
[25] Segerer S. The role of chemokines and chemokine receptors in progressive renal diseases. Am J Kidney Dis 2003; 41: S15-18.
[26] Zager RA, Johnson AC and Geballe A. Gentamicin suppresses endotoxin-driven TNF-alpha production in human and mouse proximal tubule cells. Am J Physiol Renal Physiol 2007; 293: F1373-80.
[27] Danoff TM. Chemokines in interstitial injury. Kidney Int 1998; 53: 1807-1808.
[28] Ghosh S and Hayden MS. Celebrating 25 years of NF-κB research. Immunol Rev 2012; 246: 5-13.
[29] Wynn TA and Barron L. Macrophages: master regulators of inflammation and fibrosis. Semin Liver Dis 2010; 30: 245-257.
[30] Nazinitsky A and Rosenthal KS. Cytokine storms systemic disasters of infectious diseases. Infectious Diseases in Clinical Practice 2010; 18: 188-192.
[31] Fainboim L, Cherñavsky A, Paladino N, Flores AC and Arruvito L. Cytokines and chronic liver disease. Cytokine Growth Factor Rev 2007; 18: 143-157.
[32] Anas AA, Wiersinga WJ, de Vos AF, van der Poll T. Recent insights into the pathogenesis of bacterial sepsis. Neth J Med 2010; 68: 147-152.